



Enzymatic treatment of duck hepatitis B virus: Topology of the surface proteins for virions and noninfectious subviral particles

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Abstract

The large surface antigen L of duck hepatitis B virus exhibits a mixed topology with the preS domains of the protein alternatively exposed to the particles' interior or exterior. After separating virions from subviral particles (SVPs), we compared their L topologies and showed that both particle types exhibit the same amount of L with the following differences: 1—preS of intact virions was enzymatically digested with chymotrypsin, whereas in SVPs only half of preS was accessible, 2—phosphorylation of L at S118 was completely removed by phosphatase treatment only in virions, 3—iodine-125 labeling disclosed a higher ratio of exposed preS to S domains in virions compared to SVPs. These data point towards different surface architectures of virions and SVPs. Because the preS domain acts in binding to a cellular receptor of hepatocytes, our findings implicate the exclusion of SVPs as competitors for the receptor binding and entry of virions.

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Introduction

The family of *Hepadnaviridae* with the human hepatitis B virus (HBV) as the prototype member consists of small DNA-containing enveloped viruses, which cause severe liver diseases in humans, rodents and birds (Barker et al., 1975; Ganem, 1996; Schultz et al., 2004). One characteristic feature of mammalian hepadnaviruses is the presence of three envelope proteins called large (L), middle (M), and small surface protein (S). All three proteins share the same sequence at the carboxy terminus, while M consists in addition to S of a region designated preS2 and L is further extended by the preS1 domain at the amino-terminal end. L and S exist as unglycosylated and single-glycosylated forms, whereas M appears single—as well as double-glycosylated (Bruss and Ganem, 1991; Eble et al., 1986). In contrast, the envelope of duck HBV (DHBV) contains only the two unglycosylated surface proteins S (P18) and L (P36), where consequently the S part of the latter is extended by a preS subunit. A fraction of DHBV L is phosphorylated at several

serine and threonine residues, the major phosphorylation site residing at amino acid position 118, S118 (Grgacic and Anderson, 1994; Grgacic et al., 1998). Although both members of hepatitis B viruses act similarly by producing a large amount of so-called SVPs, which contain no nucleocapsid, those empty particles show different features concerning their envelopes (Ganem, 1991; Marion, 1988; Mason et al., 1980). Where SVPs of HBV contain in contrast to HBV virions only very small amounts of L, if at all (Heermann et al., 1984), the amounts of envelope proteins of DHBV virions and SVPs disclose no differences (Bruns et al., 1998). This may be one explanation for the difficulties in separation of both DHBV particles by density gradient ultracentrifugation, which so far is only possible by using special separation techniques.

The importance of L for the infection process became evident by the identification of DHBV neutralizing epitopes in the preS region of L (Chassot et al., 1993; Chassot et al., 1994; Lambert et al., 1991) and the demonstration that the host-range specificity is also located on this part of the protein (Ishikawa and Ganem, 1995).

On the other side, it is remarkable that an about 1000-fold surplus of DHBV SVPs compared to virions usually

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detected in sera of infected hosts, fails to inhibit the infection process significantly, when the epitope composition of the envelope is expected to be comparable. Moreover, reduction of viral entry into the cell could only be accomplished by using an extremely high dose of SVPs (Bruns et al., 1998; Klingmüller and Schaller, 1993). On the contrary, addition of recombinant L protein during infection abolishes viral entry (Breiner et al., 1998). In the attempt to interpret these data, the question came up whether virions and SVPs attach to the same cellular receptor molecules during the early phase of infection or whether they enter hepatocytes using different pathways. In the latter case, one would expect distinguishing envelope features. In order to investigate the external envelope architecture, virions and SVPs of DHBV were separated from each other by a special separation technique using Urografin as a medium for steep and flat gradients (Bruns et al., 1998). Thereupon, the envelope proteins S (P18) and L (P36) of DHBV virions and SVPs could be separately analyzed.

Results

Separation of DHBV particles into virions and SVPs

Virus particles, concentrated via ultracentrifugation were separated into virions and SVPs over two alternative Urografin gradient centrifugations, where the concentrated particles were first placed on top of a 0 to 40% linear Urografin gradient and centrifuged to equilibrium with $150,000\times g$ for 18 h (Fig. 1a). Thereafter, fractions of the gradients positive for viral DNA (as an attribute of virions) and fractions negative for viral DNA but positive for L protein (predominantly detected in association with the much higher number of SVPs) were collected independently and subsequently subjected to two parallel isopycnic centrifugations in 26.5% Urografin at $150,000\times g$ for 18 h for further enrichment and separation of virions and SVPs (Fig. 1b). Fractions of the latter gradients were again analyzed for viral DNA and L demonstrating the efficiency of separation between DNA-containing virions concentrated near the bottom

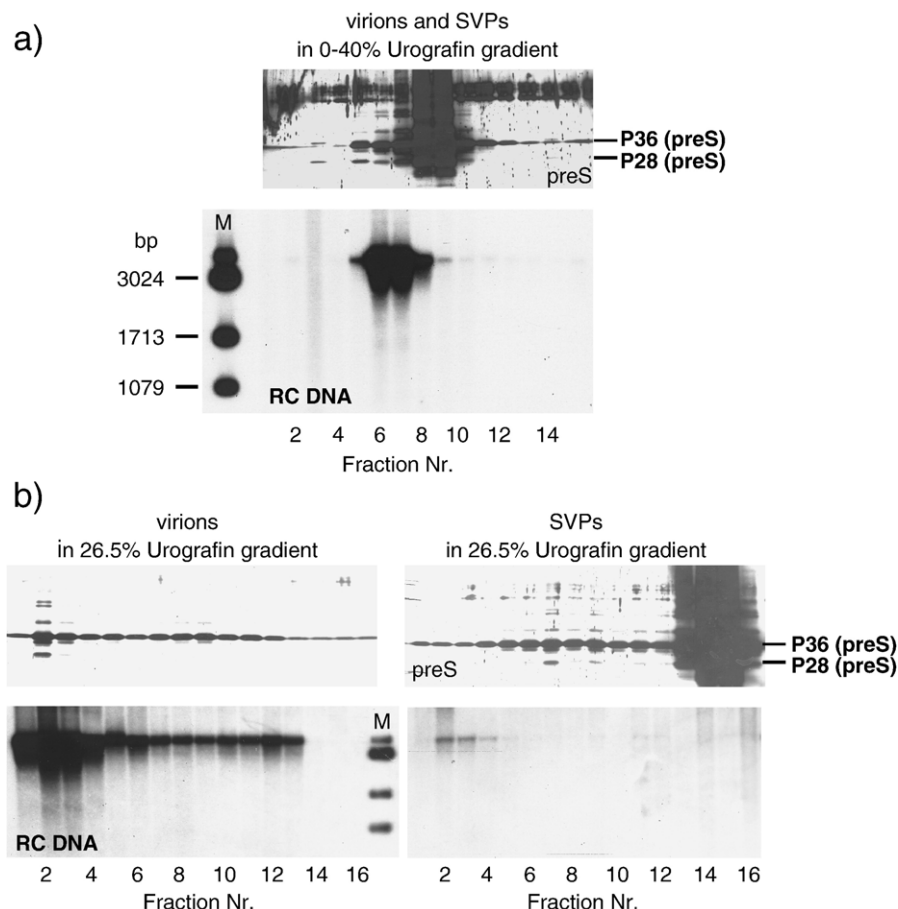


Fig. 1. Separation of DNA-containing particles (virions) and SVPs of DHBV by two successive ultracentrifugations in different Urografin gradients. (a) Western blot with an anti-DHBVpreS antibody (top) and Southern blot with a labeled DHBV-DNA probe (bottom) were performed on ultracentrifugation fractions to distinguish SVPs and virions separated by an Urografin gradient of 0 to 40%. Virions were mainly concentrated at higher densities in fractions 6 and 7, whereas most SVPs were present in fractions 8 and 9 as shown for the positions of viral DNA (bottom) or L protein (preS, top), respectively. (b) Partly purified virions (fractions 6 and 7) or SVPs (fractions 8 and 9) from the first steep gradient were collected and run in two parallel flat gradients of 26.5% Urografin. The gradient shown on the left enriched virions mainly in fractions 2 and 3 as revealed by Southern blot (bottom), whereas the second gradient on the right demonstrated the accumulation of SVPs in fractions 14 and 15 in the Western blot (top). RC DNA, relaxed circular DNA; P36 and P28, the large surface protein (L) and the cleavage product of it, most prominently seen in SVP preparations because of its 1000-fold surplus over virions; M, digested pMaD-26 plasmid DNA.

of the flat gradient (Fig. 1b, left), whereas SVPs with slightly less densities remained at the top of the second Urografin gradient run in parallel (Fig. 1b, right). Table 1 gives a typical example of the enrichment for virions and SVPs by the two-step gradient ultracentrifugation. The virion fraction was 10-fold enriched for virions and at the same time the amount of SVPs in that fraction was approximately reduced by a factor of 10. So, the overall composition of that fraction was about 100-fold enriched for virions when compared to the virion–SVP-ratio in the mixture (although it should be noted that in this sample the accuracy of the measurement of the amount of L/μl as an evaluation for SVPs was rather low because the signal was close to background level). The SVP fraction was more than 30-fold enriched for SVPs and the amount of virions in that fraction was reduced about 120-fold at the same time. So, the overall composition of this fraction was nearly 4000-fold enriched for SVPs when compared to the starting mixture.

Virions and SVPs of DHBV contain the same proteins and in similar amounts within the viral envelopes

In general, no different composition of the viral envelope proteins was evident within purified virions and SVPs. The following experiment (Fig. 2) exhibited the similar composition of the envelopes of both entities. When 1×10^{10} virions were compared with 1×10^{10} SVPs, only minor quantity differences could be seen in Western blots of electrophoretically separated DHBV envelope proteins. This was also true for the cellular heat shock protein HSC70, whose presence in DHBV particle preparations (unpublished observation) and role in the activation of the viral reverse transcriptase (Beck and Nassal, 2003) were demonstrated earlier.

Proteolytic cleavage products of viral envelope proteins

SVPs were now selected for the analysis of the proteolytic cleavage products after the digestion of intact particles with chymotrypsin. Enzymatically treated and untreated SVPs were separated by PAGE to be investigated in subsequent Western blots with different monoclonal and polyclonal antibodies (Fig. 3, bottom). The polyclonal antisera were introduced in this experiment as controls because of their ability to detect all

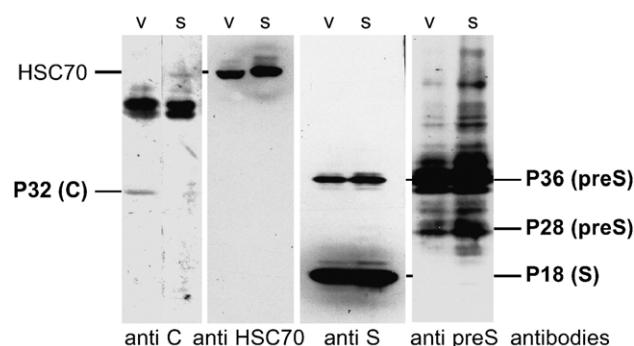


Fig. 2. Examination of the envelope proteins of DNA-containing DHBV particles and particles devoid of DNA. 10^{10} virions (V) and SVPs (S) each were four times side by side in parallel electrophoretically separated. After blotting the membrane, sections were cut and incubated with anti-C, anti-S, anti-preS and anti-HSC70 antibodies. No crucial differences could be demonstrated in the amount of envelope proteins between virion and SVP fractions. The large surface protein L (P36) and its cleavage product (P28) were detected with an antibody recognizing the preS part of this component only, whereas the small surface protein S (P18) was demonstrated with an antibody reacting with the S subunit present in both envelope proteins. Additionally, the cellular heat shock protein HSC70 was found to be present in similar amounts in the virion as well as SVP fractions. The viral core protein P32 (C) was only present in the nucleocapsid-containing virions. The two upper bands, detected with anti-C, are contaminating proteins of unknown origin.

possible fragmented products in addition to the intact S and L proteins (Figs. 3d and e). The proteolytic cleavage products detected by the epitope-specific monoclonal antibodies (Mabs) 900, 1D2, and 7C.12 (Chassot et al., 1993; Guo and Pugh, 1997a; Lambert et al., 1990) or by polyclonal anti-S or anti-preS antibodies are depicted in the upper segment of Fig. 3. Their variety suggests that the digest was either incomplete or the conformation of envelope proteins within the membrane of intact particles differed in a way that chymotrypsin recognition sites were alternatively accessible or hidden from enzymatic attack, because different cleavage products of P28 (16 kDa and 17 kDa) and P36 (21 kDa and 26 kDa) were observed. At the domain structure of L, all possible chymotrypsin recognition sites are marked with the exception of a section between the two trans-membranous regions TM1 and TM2 (Eble et al., 1986). This area, which is situated near the amino-terminal end of P18 as well as of the corresponding S subunit of P36, should be protected against a direct interaction with any enzyme because of its internal localization. Mabs 900 and ID2 detected a chymotrypsin cleavage fragment with a relative molecular weight of 18 kDa (Figs. 3a and b). This fragment is obviously part of the preS domain of L as Mabs 900 and ID2 recognize only epitopes within that subunit. Mab 7C.12 instead as the anti-S antiserum detects a cleavage fragment of similar size (17 kDa) but as this antibody recognizes an epitope within the S domain of P36 these two chymotrypsin cleavage products must be of different origin (Figs. 3c and d).

Another fragment with a relative molecular weight of 21 kDa could be detected with all other antibodies (Figs. 3b–e), but not with Mab 900 (Fig. 3a). The appearance of such a fragment can be most plausibly explained, when parts of the amino-terminus as well as of the carboxy-terminus of L were removed by

Table 1

Enrichment of DHBV and SVPs as monitored by measuring the amount of viral genome equivalents (vge)^a and L protein molecules^b

Virus sample	vge/ml	L/ml
Mixture (start)	3.7×10^8	3.8×10^{11}
Purified virions	3.9×10^9 ^c	4.1×10^{10}
Purified SVPs	$<3.1 \times 10^6$	1.2×10^{13} ^d

^a Calculated by dot blot experiments using a radioactively labeled DHBV-DNA probe.

^b Calculated in a BioRad Fluor-S™ Multi Imager with the program “Quantity One” and an anti-preS antiserum.

^c Corresponding to 12.72 pg/μl.

^d Corresponding to 47.78 ng/μl.

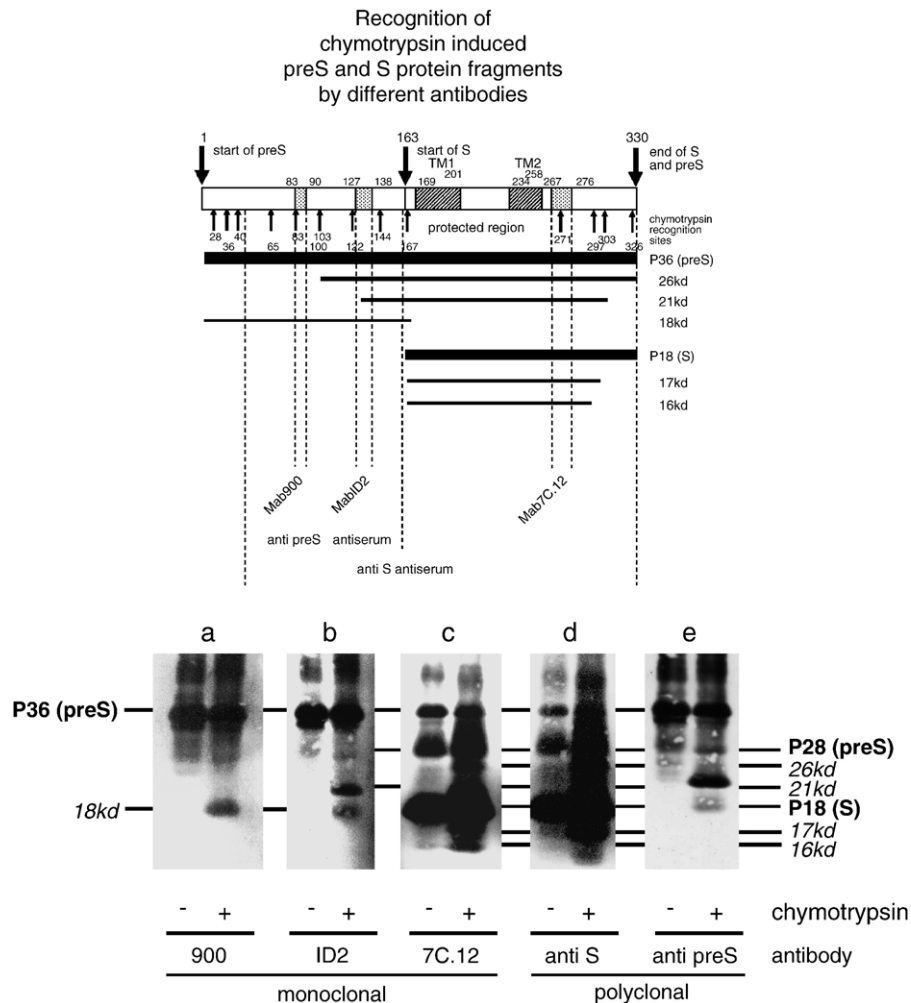


Fig. 3. Chymotrypsin recognition sites and the identification of the potential chymotrypsin-induced preS and S protein fragments by different antibodies. Purified SVPs were divided to be incubated with chymotrypsin (+) or not (-). After enzyme treatment, viral proteins were disrupted, separated by PAGE in five parallel pairs of chymotrypsin-treated and mock-treated samples (a–e) and blotted onto a membrane. For Western blots, the membrane was cut and either incubated with the Mabs 900 (a) or 1D2 (b) for the identification of epitopes on the preS region, or with Mab 7C.12 (c) for the recognition of an epitope within S. In addition, the polyclonal anti-S and anti-preS antisera were used recognizing epitopes across the entire S and preS domains respectively. The upper part presents graphically the domain structure of L (open bar), where the dotted areas mark the epitopes detected by the different Mabs and the striped boxes the two known transmembraneous regions (TM1 and TM2) enclosing a region most probably protected against enzymatic attacks. The fat arrows on top designate the starting points for translation of P36 and P18, respectively, and the identical termination point for both proteins, whereas the small arrows below L illustrate the possible recognition sites for chymotrypsin with the exception of the protected region between TM1 and TM2. The small numbers above and below elucidate different amino acid positions. The bars below L illustrate the original viral proteins (strong bars) and their possible cleavage products (thin bars) as detected by the different antibodies. The naturally occurring cleavage product of L, P28, as visible in blots with untreated SVPs (c, d and e), is not introduced in the diagram.

chymotrypsin, leaving a fragment between aa 103 and 297 (for clarity not shown) or aa 122 and 303 intact (see the 21 kDa fragment drawn in the sketch on top of Fig. 3). If this is true and the conformation allows access, S should also be processed at aa 297 and/or 303 to give rise to fragments of 16 and/or 17 kDa. In this respect, it was not surprising to detect with Mab 7C.12 besides the original P18 some cleavage products with relative molecular weights of 16 kDa and 17 kDa (Fig. 3c), obviously representing carboxy-terminally truncated polypeptides originating from P18. On the other side, there was the unexpected observation that more than half of L remained intact and could not be manipulated enzymatically (Figs. 3 a, b and e). If this was not because of an incomplete digest, this finding supports data that showed that the preS domains of L were partly hidden

inside the envelope and partly located on the outside of the viral particles (Grgacic and Anderson, 1994; Guo and Pugh, 1997b). Figs. 3c and d also show that P28, a cleavage product of L (Fernholz et al., 1993), always detectable in infected primary duck hepatocytes (PDHs) and in purified virus (Bruns et al., 1998), also remained at least partially unaffected by the enzyme. This product, which is for reason of clarity not inserted in the map, can be detected by both polyclonal antisera (Figs. 3d and e) as well as all three Mabs, which maps the protein to include aa 83 to 276 of L. Comparing the Mab reaction in parallel it was evident that only Mab 7C.12 was able to strongly detect P28 (Fig. 3c). At the same time, the detection of the full length P28 after chymotrypsin digestion suggests a localization that protects it from enzymatic attack.

In purified virions the majority of preS domains are located on the outside

Using the anti-DHBVpreS antiserum, which strictly recognizes the amino-terminal end of L, we wanted to investigate differences in the exposure of preS domains of virions and SVPs. Purified intact particles from DHBV positive duck serum were separately digested with chymotrypsin or mock digested. After the digest particles were subjected to ultracentrifugation in a 0–70% sucrose gradient to dispose of detached peptides. Fractions were drawn and assayed for the preS domain in protein dot blotting and Western blotting (Fig. 4). Virions and SVPs behaved strikingly different in this assay. As was to be expected, after digestion with chymotrypsin, almost no L protein could be detected either on the surface of virions or SVPs, when judged against virus particles treated with buffer only (Figs. 4a and b, top). This result argues for a complete removal of surface preS domains by digestion at aa position 167 (compare Fig. 3). The Western blots of virions stripped off the external preS domains showed surprisingly that essentially no P36 was left, whereas

cleavage products of 26, 21 and 17 kDa could be detected (Fig. 4a, bottom right). Unlike virions, Western blotting of chymotrypsin digested SVPs revealed significant amounts of intact P36, which must have been buried within the membrane or particle. Besides P36, minor amounts of 26 kDa and 21 kDa cleavage products were evident (Fig. 4b, bottom right). Taken together, the most important finding after treatment of intact purified viral particles with chymotrypsin was the general loss of the preS domain of P36 in virions, whereas more than 50% of this subunit in SVPs remained not involved in the cleavage. This result gives good evidence to argue for a general difference in the topology of envelope proteins of virions and SVPs with almost all of the preS domains exposed on the virion exterior but more than half of them buried within the interior of SVPs.

The phosphorylated preS domain is completely exposed on the exterior of virions but partially hidden in SVPs

The preS domain of L bears a major phosphorylation site at aa 118 (S118), whose phosphorylation is responsible for a

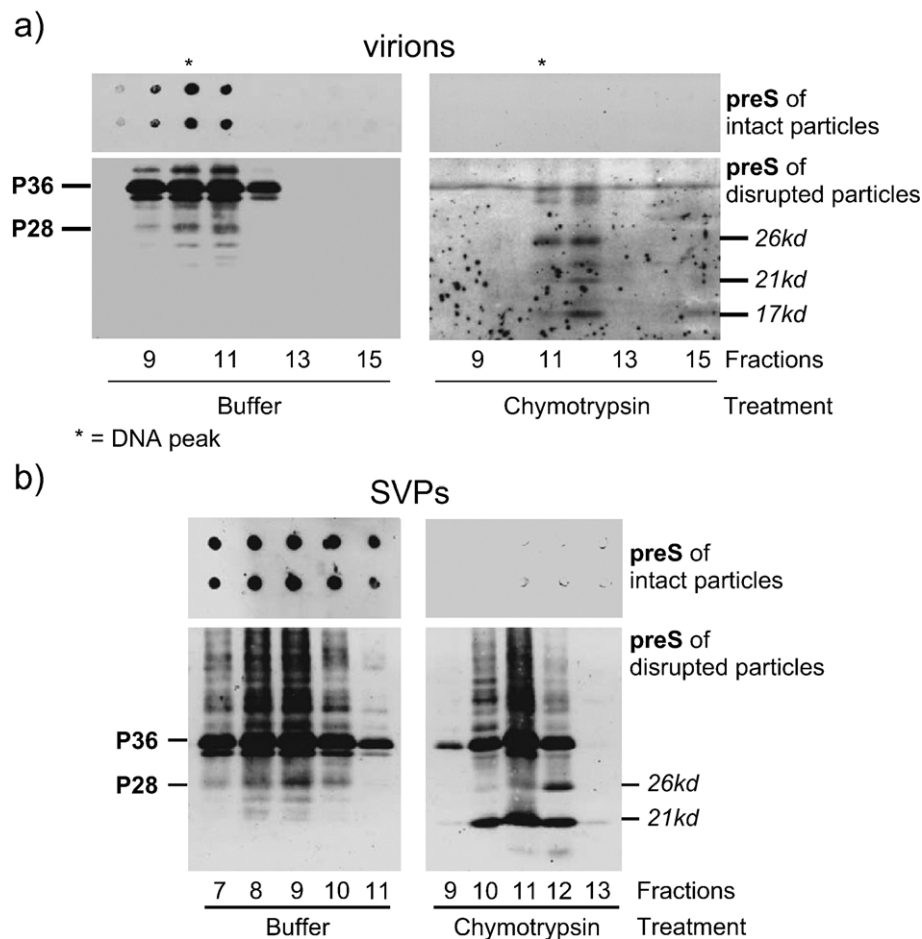


Fig. 4. Differential exposure of preS domains on the exterior of virions and SVPs. Purified virions (a) or SVPs (b) were either incubated in buffer (left) or in solution containing chymotrypsin (right). After the digest particles were subjected to separation in a 0 to 70% sucrose gradient. One-microliter aliquots of particle-containing fractions were directly dotted onto membranes (upper strips), whereas the remaining fractions were subjected to PAGE and thereafter blotted onto membranes (lower blots). Then, strips and blots were treated with the anti-preS antibody for the detection of the amino-terminal region of P36. The asterisks above the strips at fraction 10 of buffer-treated (left) as well as fraction 11 of chymotrypsin-treated virions (right) single out the DNA peaks measured in parallel (not shown). The products with relative molecular weights of 17, 21, and 26 kDa are cleavage products of L detected with the anti-preS antibodies.

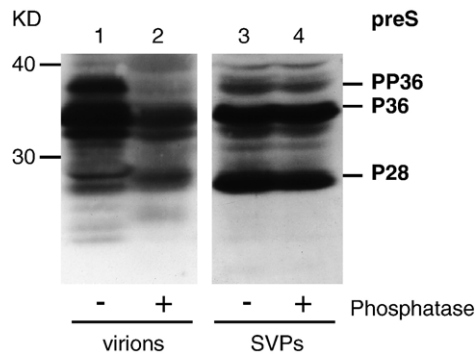


Fig. 5. Comparison of the phosphorylated surface proteins of virions and SVPs from DHBV. Purified virions or SVPs were either left untreated (1 and 3) or were treated with acid phosphatase (2 and 4) to remove the phosphates from the outside accessible phosphorylation of L. Enzymatically digested particles were subjected to PAGE and Western blotting with the anti-preS antibody. The phosphorylated form of L (PP36) runs slightly above P36. Treatment of virions with phosphatase removed PP36; a partial dephosphorylation of P28 is also visible (not indicated, compare the corresponding regions in lanes 1 and 2). By contrast, SVPs treated with phosphatase preserved PP36.

retarded SDS-PAGE migration behavior of Phospho-L (PP36) to a position of 37 kDa. PP36 is thus easily distinguishable from P36 in Western blotting (Grgacic et al., 1998). PP36 of serum derived viral particles has been shown to be resistant to phosphatase digestion in the absence of detergents (Grgacic and Anderson, 1994). Considering the large excess of SVPs over virions in serum, this has to hold true for SVPs; it, however, may be different for virions. If almost all of the preS domains of virions are actually exposed on the outside of the particle, it would be expected that phosphatase treatment of intact virions is able to remove phosphorylation at S118 together with the aberrant migration band PP36.

We compared purified untreated virions and SVPs (Fig. 5, lanes 1 and 3) with phosphatase-treated virions and SVPs in Western blots (Fig. 5, lanes 2 and 4) and could show that PP36 of virions was almost completely lost during the phosphatase treatment (Fig. 5, lane 2), whereas PP36 of SVPs remained visible (Fig. 5, lanes 3 and 4). This result adds to the evidence for a different topology of L in virions and SVPs.

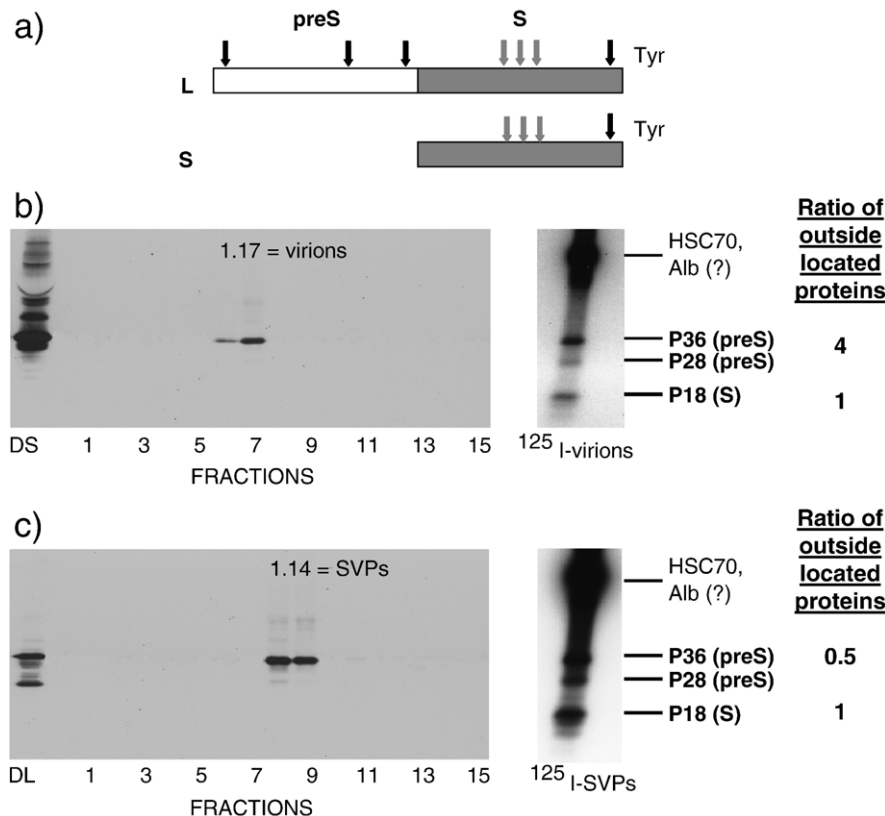


Fig. 6. Analysis of the surface components of virions and SVPs accessible for iodination. (a) Position of tyrosines, Tyr (arrows), as the target for iodine in DHBV L and S; the tyrosines in the center of the S domain (grey arrows) are most probably protected against iodination because of their inner localization. (b) Western blot of virion fractions after gradient purification (left). Fractions 6 and 7 of the virion gradient were combined and particle surfaces labeled with radioactive iodine. Labeled particles were again subjected to PAGE. The gels were dried and exposed to X-ray film (right). (c) Western blot of SVP fractions after gradient purification (left). Fractions 8 and 9 were combined and particle surfaces labeled with radioactive iodine. Labeled particles were again subjected to PAGE. The gels were dried and exposed to X-ray film (right). The strongly labeled component/s shown on top of both gels could represent the cellular heat shock protein (HSC70) generally co-purified with DHBV particles or co-purified serum albumin (Alb) eventually adhered to the outside of the particles. The positions of the DHBV envelope proteins L (P36 and P28) and S (P18) are shown on the right side together with the ratios of measured radioactivity demonstrating the degree of their external localizations. The Western blot representing L and the dried gel containing the iodinated proteins of the virions (a) were exposed exactly tenfold longer to get an adequate darkening of bands for comparison with the corresponding samples of SVPs (b). DL and DS, DHBV marker proteins from DHBV positive duck liver and serum, respectively.

External iodination revealed a different ratio of accessible tyrosines on preS and S domains of virions and SVPs

The sequence of L harbors 7 tyrosines (Y), Y10, Y138, Y179, Y258, Y274, Y293 and Y363, that can theoretically be labeled with radioactive iodine (Fig. 6). Y10, Y138 and Y179 are positioned on the preS domain; Y258, Y274 and Y293 are found within the sequence between TM1 and TM2. Because this region is believed to have an internal orientation, these three sites should not be accessible for iodination without prior disruption of the particles. Finally Y363 is located close to the C-terminal end of the S domain. Radioactive labeling of all four theoretically from the outside accessible tyrosines should result in an L-to-S-signal ratio of 4:1. This holds true for particles where the preS domain is exclusively located on the outside. Internal localization of preS domains should consequently shift this ratio towards a lower L-signal. For the following iodination experiment, virions and SVPs, each enriched by steep and flat Urografin gradients, were again re-purified over a 0–40% Urografin gradient to obtain highly purified intact viral particles for their external labeling. A preliminary investigation of aliquots of the fractions by Western blots revealed the grade of purity for both particles, where in the virion-containing fractions no SVPs at a density of 1.14 g/cm^3 could be detected, but only particles migrating to the expected position of 1.17 g/cm^3 and vice versa (Figs. 6b and c, left). The virion gradient fractions 6 and 7 with an average density of 1.17 g/cm^3 (Fig. 6b, left) and the SVP gradient fractions 8 and 9

with an average density of 1.14 g/cm^3 (Fig. 6c, left) were collected, concentrated by ultracentrifugation, and the pellets were resolubilized in phosphate buffer before iodination with the IODO-GEN procedure, which, as verified for another enveloped virus (Bruns et al., 1983; Bruns et al., 1986), exclusively labeled the viral outside. After the labeling procedure, viral particles were disrupted and separated by PAGE to reveal the amount and identity of labeled proteins of virions (^{125}I -virions; Fig. 6b, right) and SVPs (^{125}I -SVPs; Fig. 6c, right). The analysis of the radioactive signals of both particles revealed a similar pattern of proteins. According to their co-migration with DHBV envelope proteins detected by Western blot, radioactively labeled components could be identified as L (P36 and P28) and S (P18). Another dominantly labeled band consisting of at least one protein at a position of about 70 kDa could originate from co-purified and labeled HSC70 protein, whose presence in purified DHBV particle preparations was demonstrated in an earlier experiment (see Fig. 2). As the exact localization of HSC70 is not known, we can only speculate how far the radioactive iodine molecules were able to penetrate the membrane to come into close contact with this component, but obviously not so far as to label the core protein. Alternatively, the secretion of HSC70 as part of the membrane of exosomes has been described (Broquet et al., 2003; Tytell, 2005). Preliminary experiments in our laboratory have shown that HSC70 originating from the supernatants of the chicken hepatoma cell line LMH (Kawaguchi et al., 1987) runs in the same fractions of Urografin gradients as DHBV virions

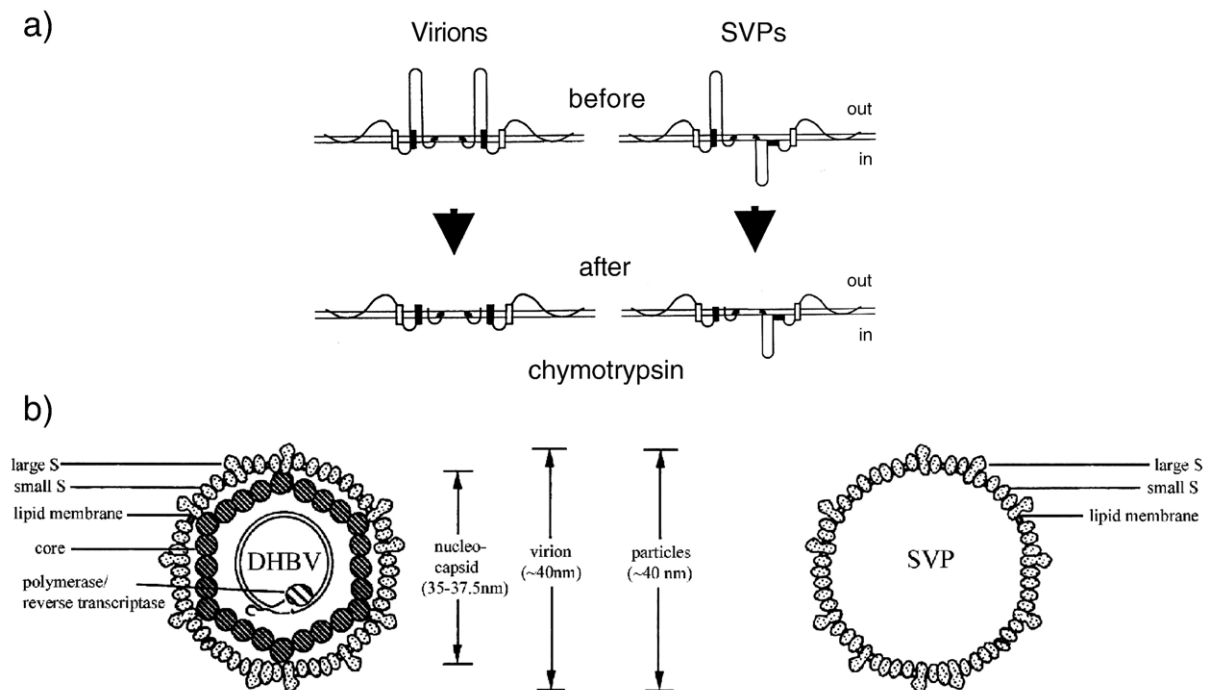


Fig. 7. Model for the topology of the large envelope proteins of DHBV virions and SVPs. (a) Complete accessibility of chymotrypsin leads to the removal of the preS loop from L in virions, whereas most of the amino-terminal part of this component is protected against the enzymatic attack in SVPs. (b) The presence of the nucleocapsid in virions might deliver an explanation for the improbability of an internal localization of L in virions (left), whereas the absence of this obstacle allows preS domains to be positioned within the viral membrane.

and SVPs. Thus, copurified exosomes carrying HSC70 in their membrane could be responsible for the iodinated 70 kDa species. Additionally, serum albumin secreted from PDHs and eventually sticking to the surface of the particles could account for this band. Although not precisely known for ducks, both HSC70 and albumin carry several tyrosines as candidates for iodination. HSC70 is an extremely well conserved protein exhibiting 15 tyrosines; human albumin carries 19 and that of chicken, 20 tyrosines.

Virion protein bands were excised and their amount of radioactivity measured. Comparison of signal intensities resulted for virions in an L-to-S-ratio of 4:1 and for SVPs in an L-to-S-ratio of 0.5:1. The L-to-S-ratio of radioactively iodinated virions nicely reflects the expected ratio, when all of the preS domains are exposed on the virion surface (see above, Figs. 6 and 7). The low L-to-S-ratio of labeled SVPs points towards a general difference in the topology of preS domains of SVPs compared to virions. The fact that the radioactive signal for L was lower than that for S could indicate that almost all preS domains including some of the L C-termini are buried within the particle and not accessible for iodination. We take this as additional proof for the different surface architectures of DHBV virions and SVPs.

Discussion

The relatively successful separation performance using gradient ultracentrifugation for HBV particles facilitated the discovery that the surfaces of HBV virions and SVPs differ strongly in as far as L is missing in the latter (Ganem, 1991; Marion, 1988; Mason et al., 1980). Meanwhile, potential differences in the surface architecture of DHBV virions and SVPs with both particles exhibiting the same L-to-S-ratio remained enigmatic because of the difficulty to separate them. For this reason, mixed DHBV particle preparations were used up to now for studies of the viral envelope, which can, because of the large excess of SVPs over virions, more or less be interpreted as SVP-preparations (Grgacic and Anderson, 1994; Grgacic and Anderson, 2005; Grgacic and Schaller, 2000). We employed here a special multiple step ultracentrifugation protocol using subsequent steep and flat Urografin gradients, which made the separation of both particle types for an examination of the viral surface proteins possible (Bruns et al., 1998). Although on the first view infectious and non-infectious particles revealed no diversity in the composition of their envelope components, the absolute amounts of S and L, we were able to accumulate evidence for a different orientation of preS domains on the surfaces of virions and SVPs with almost all of them exposed on the virion exterior but the majority of them buried within the SVP. First, we showed that almost all preS domains of virions exposed to chymotrypsin were digested, whereas a large amount of the respective domains of SVPs remained intact. Secondly, we could illustrate that the phosphorylation of L at S118 could be removed by incubating intact virions with phosphatase, while SVPs remained phosphorylated. And finally, specific labeling of tyrosines on the outside of undisrupted particles with iodine-125 disclosed a

significantly higher ratio of exposed preS domains to S domains in virions compared to SVPs. Considering the usual high surplus of SVPs over virions, the observed discrepancies between the published data and our findings were not generally surprising.

As a summary of our results, we extend a model first presented by Guo and Pugh (1997b): our data clearly demonstrate that most, if not all, preS domains in virions are located on their surface, whereas the majority of them in SVPs are hidden inside (Fig. 7a). Treatment of virions with chymotrypsin leads to a nearly total removal of all preS domains of the particles, while incubation of SVPs with that protease leaves most of L unaffected. When the different topology of L was first discovered by Guo and Pugh (1997b), it was suggested that the preS loop of L will be translocated during particle morphogenesis in a manner of spring-loaded mechanism to their surface. According to our results, the initial trigger for this mechanism could be mechanical or electrostatic forces due to the envelopment of the nucleocapsid, leaving only a very limited space between nucleocapsid and envelope (Fig. 7b, left). On the contrary, the missing nucleocapsid in SVPs might deliver a plausible explanation for the likely localization of preS domains inside the empty particles (Fig. 7b, right). In summary, these findings let us conclude that, although the total quantities of surface proteins are nearly identical in infectious and non-infectious particles, the surface architectures of both particles are markedly different; where in virions the majority of preS-loops is localized on the outside, they are much less numerous exposed on the surface of SVPs. In this respect, DHBV SVPs resemble the surface of HBV SVPs, where L is not present at all. In addition, this point of view combined with the knowledge that the attachment of DHBV virions to host cells is accomplished by the contact of the preS domain with the cellular receptor gp180/carboxypeptidase D (Breiner et al., 1998; Ishikawa et al., 1994; Kuroki et al., 1994; Kuroki et al., 1995; Li et al., 1996; Tong et al., 1995, 1999; Urban et al., 1998), could provide an explanation why the usual large surplus of SVPs in DHBV does not inhibit infectivity (Bruns et al., 1998; Klingmüller and Schaller, 1993).

Finally, since gp180 is not exclusively expressed on hepatocytes, it remains to speculate whether the viral envelope architecture bears the necessary information for the penetration of the hepatocyte membrane. A multi-receptor complex as proposed by Li et al. (2004) could possibly discriminate between a higher and lower amount of exposed preS domains. Otherwise, when viral entry does not distinguish between virions and SVPs, as imposed by equal binding of both particle types on the hepatocellular membrane (Funk et al., 2004), one could imagine a mechanism later in the process of infection. As for viral entry, it has been shown that it proceeds by endocytosis (Funk et al., 2004) followed by viral escape from late endosomes after exposure of a translocation motif (TLM) probably by proteolytic cleavage (Stoeckl et al., 2006). Competition of SVPs for the protease activity and thus inhibition of efficient virus cleavage could be counteracted by the internal localization of preS domains.

So far it remains unresolved whether virions and SVPs of DHBV attach to the same receptor molecules or whether they follow different pathways, maybe not only in liver parenchymal but also in non-parenchymal cells like fat-storing (Ito) cells or liver-specific macrophages (Kupffer cells) and liver-sinusoidal endothelial cells (LSECs) (Breiner et al., 2001).

Materials and methods

Plasmid and antibodies

DHBV pMaD-26 plasmid DNA (Fernholz et al., 1993) was alternatively cleaved with the restriction enzymes *EcoRI* and *HindIII* and mixed in a ratio of 1:2 to obtain fragments of 3024, 1713, 1079 and 244 base pairs (bp). The Mabs 900 and ID2 detecting epitopes within preS of the L protein between aa 83–90 and aa 127–138, respectively (Chassot et al., 1993), the Mab 7C.12 identifying an epitope within the S domain between aa 267 and 276 (Pugh et al., 1995), the polyclonal anti-S (Klingmüller and Schaller, 1993), the anti-preS or the anti-C antisera (Lambert et al., 1990; Schneider and Will, 1991) were alternatively used for fragment analysis of the DHBV surface proteins or the examination of intact DHBV particles. Antibodies against HSC70 were obtained from Sigma (Deisenhofen, Germany).

Purification of virus

Virus purification was performed exactly as described earlier (Bruns et al., 1998). In brief, virus particles were isolated from sera of DHBV carrier ducks, which were infected with strain DHBV-3 2 days after birth. Virus-containing material was clarified by low-speed centrifugation and thereafter layered onto 20% sucrose cushions in GNT buffer (0.2 M glycine, 0.2 M NaCl, 0.02 M Tris and 0.002 M EDTA, pH 7.5) and centrifuged for 1 h in a SW55 rotor (Beckman, Munich, Germany) at 200,000×g. The pellets were dissolved in GNT buffer and placed on top of a 0 to 40% linear Urografin (3,5-diacetamido-2,4,6-tri-iodobenzoic acid, Schering AG, Berlin, Germany) gradient and centrifuged to equilibrium with 150,000×g for 18 h. Fractions of the gradients were drawn from the bottom of the tubes and analyzed for viral DNA (as a trait of virions) and viral large envelope protein (L) content. To separate particles of different densities, the fractions of the first centrifugation step enriched for viral DNA or devoid of viral DNA but enriched for viral proteins (a characteristic of SVPs) were collected independently and were subsequently subjected to two parallel isopycnic centrifugations in 26.5% Urografin at 150,000×g for 18 h for further enrichment and separation of virions and SVPs. Fractions of the second gradients were again analyzed for viral DNA and L. Those positive for viral DNA or devoid of viral DNA but positive for L were collected, diluted in GNT buffer, pelleted by ultracentrifugation at 200,000×g and stored at −70 °C as purified virions or purified SVPs, respectively. Alternatively, before iodination, particles were once more run through a 0–40% Urografin gradient for further

purification. All gradient centrifugation steps were carried out at 4 °C using a SW55 rotor (Beckman).

Calculation of the amount of virus particles

After blotting the intact particles onto nitrocellulose membranes, the number of viral genome molecules as measured by dot blot hybridization and quantified by using standards of cloned DHBV DNA (Fernholz et al., 1993; Lambert et al., 1990) was considered equivalent to the number of virions since viremic sera seem to contain very few defective genomes (Jilbert et al., 1996). The amount of SVPs was estimated by comparison of the L content of purified SVPs with that of a defined number of virions in polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting with an anti-DHBVpreS antiserum. The same signal intensity for virions and SVPs after the immune reaction with anti-DHBVpreS, measured in a BioRad Fluor-S™ MultiImager with the program “Quantity One”, was taken as evidence for roughly identical numbers because the two types of viral particles are similar in size and seem to contain the same ratio of L to S proteins (Bruns et al., 1998).

Enzyme treatment of purified viral particles

Separated purified DHBV particles were treated with 100 µg/ml chymotrypsin in phosphate-buffered saline (PBS), pH 7.5, for 0.5 h at room temperature as described earlier for purified lymphocytic choriomeningitis (LCM) virus (Bruns and Lehmann-Grube, 1984). In order to remove degraded products, digested virus particles were ultracentrifuged in a 0 to 70% sucrose gradient with 200,000×g for 4 h at 4 °C in a SW55 rotor (Beckman). For dephosphorylation, purified chymotrypsin-treated or mock-treated viral particles were, after removal of degraded products, alternatively incubated with 1 U/ml acid phosphatase or buffer only according to a protocol already described (Grgacic and Anderson, 1994). All enzymes were purchased from Sigma.

Preparation and analysis of DHBV DNA

Fractions of gradient centrifugations were diluted 5-fold and viral particles were pelleted by ultracentrifugations. Pellets were lysed with 1% NP40 and 0.5% sodium dodecyl sulfate (SDS; Sigma) in 50 mM Tris–HCl (pH 8.0) containing in addition 1 mM EDTA and 100 mM NaCl, and digested with 500 µg/ml proteinase K overnight at 37 °C. Viral DNA was extracted with phenol/chloroform and precipitated with ethanol. The DNA was dissolved in TE buffer and subjected to 1% horizontal agarose gel electrophoresis, followed by transfer onto Hybond N membrane (Amersham Buchler, Braunschweig, Germany) and UV cross-linking. For detection of virions, DHBV-3 plasmid DNA was labeled with [γ -³²P] dCTP (Megaprime, Amersham Buchler) and used for hybridization at about 10⁶ CPM/ml. The same hybridization protocol was applied to DNA dot blots, where a serial dilution of DHBV-3 containing plasmid DNA was used as a

mass standard. Specifically bound radioactivity was quantified using a Fuji Bas 2000 Phosphor Imager (Raytest, Essen, Germany).

Detection of DHBV envelope proteins

Purified virions as well as SVPs were dissolved by boiling in a double concentrated disruption buffer containing 4% SDS and 10% 2-mercaptoethanol and proteins were separated by PAGE essentially according to the method published by Laemmli (1970) in 5 to 20% gradient slab gels. For Western blot analysis, proteins separated by electrophoresis were transferred to PVDF membranes (BioRad Laboratories, Hercules, USA) and viral antigens were detected by different rabbit anti-viral antisera or by Mabs directed against epitopes within S and preS of the viral envelope proteins followed by peroxidase-conjugated goat anti-rabbit or anti-mouse sera (Dianova, Hamburg, Germany). The bands were visualized by the ECL procedure according to the manufacturer's protocol (Pierce, Rockford, USA). For labeling of the surfaces of intact particles, iodination was performed according to the IODO-GEN (Pierce, Rockford, U.S.A.) procedure (Fraker and Speck, 1978) in 0.2 M phosphate buffer, pH 7.5, a method formerly intensively studied with the external iodination of purified lymphocytic choriomeningitis virus (Bruns et al., 1983, 1986). In brief, aliquots of 200 µg IODO-GEN, dissolved in 20 µl chloroform, were adsorbed by air-drying to the bottom of 12×75 mm glass tubes. To such prepared glass vessels, purified DHBV virions or SVPs in 90 µl of 0.2 M phosphate buffer (pH 7.5) and 500 µCi/µg of Na¹²⁵I were applied. After a reaction time of 1 min at room temperature (Amersham Buchler, Braunschweig), the iodinated compounds were removed and particles were separated from free radioactive iodine by ultracentrifugation through sucrose gradients as described above. Then, the virus-containing fractions were collected, diluted 1:10 and precipitated with 10% trichloroacetic acid followed by two washing steps with ice-cold ethanol. Iodinated samples were detected by the exposure of vacuum-dried gels to X-ray films at –70 °C, the signals being intensified as described (Laskey and Mills, 1977). Bands corresponding in size to P36 and P18 were excised and measured for their content of radioactivity with a γ-ray scintillation counter (BF Gammascint 5300, Berthold, Wildbad). In the figures of this publication, naturally occurring viral proteins are presented in bold letters, whereas enzymatically formed products are shown in italics.

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